Immobilization of Glucose Oxidase on Modified-Carbon-Paste-Electrodes for Microfuel Cell

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ABSTRACT

Glucose oxidase (GOx) is being developed for many applications such as an implantable fuel cell, due to its attractive property of operating under physiological conditions. This study reports the functional immobilization of glucose oxidase onto polyaniline-nanofiber-modified-carbon-paste-electrodes (GOx/MCPE) as bioanodes in fuel cell applications. In particular, GOx is immobilized onto the electrode surface via a linker molecule (glutaraldehyde). Polyaniline, synthesized by the interfacial polymerization method, produces a morphological form of nanofibers (100-120 nm) which have good conductivity. The performance of the polyaniline-modified-carbon-paste-electrode (MCPE) was better than the carbon- paste-electrode (CPE) alone. The optimal pH and temperature of the GOx/MCPE were 4.5 (in 100 mM acetate buffer) and 65 °C, respectively. The GOx/MCPE exhibit high catalytic performances (activation energy 16.4 kJ mol^{-1}), have a high affinity for glucose (K_m value 37.79 μM) and can have a maximum current (I_{max}) of 3.95 mA. The sensitivity of the bioelectrode also was high at 57.79 mA mM^{-1} cm^{-2}.

Keywords: *biosensor; carbon-paste-electrode; glucose oxidase; glutaraldehyde; nano-fiber polyaniline*

ABSTRAK

Glukosa oksidase (GOx) adalah enzim yang banyak dikembangkan untuk aplikasi sel bahan bakar yang dapat diimplantasi. Hal ini karena aktivitas katalitiknya yang dapat berlangsung pada kondisi fisiologis. Penelitian ini melaporkan imobilisasi fungsional GOx pada elektroda pasta-karbon termodifikasi nanoserat polianilin (GOx /EPKT) sebagai bioanoda untuk aplikasi sel bahan bakar. Imobilisasi GOx pada permukaan elektroda dalam penelitian ini dilakukan dengan menggunakan glutaraldehid. Adapun polianilin yang digunakan untuk memodifikasi elektroda disintesis menggunakan metode polimerisasi permukaan untuk menghasilkan polianilin nanoserat (100-120 nm) yang memiliki konduktivitas tinggi. Hal ini ditunjukkan pada hasil bahwa elektroda pasta-karbon yang dimodifikasi polianilin nanoserat (EPKT) memiliki daya hantar listrik yang lebih baik dibandingkan elektroda pasta karbon tanpa modifikasi (EPK). Adapun kinerja bioanoda GOx/EPKT optimum dilakukan pada pH 4,5 dalam buffer asetat 100 mM dan suhu 65 °C. Bioanoda ini memiliki unjuk kerja katalisis yang tinggi (energi aktivasi 16,4 kJ mol^{-1}), afinitas glukosa yang tinggi (K_m 37,79 μM), nilai arus maksimum yang tinggi (I_{max} 3,95 mA), serta sensitivitas elektroda yang tinggi (57,79 mA mM^{-1} cm^{-2}).

Kata Kunci: *biosensor; elektroda pasta-karbon; glukosa oksidase; glutaraldehid; polianilin nano-serat*

INTRODUCTION

Enzymes are attractive to use as fuel cell electron shuttles due to their extremely high substrate specificity. Additionally, the ability of enzymes to utilize biologically derived fuels such as e.g. glucose, fructose, lactose, ascorbate, dopamine, and alcohols, along with ubiquitous O_2 as the biooxidant are the reasons of using enzymes in an array application of biofuel cells. Also, enzyme production is relatively inexpensive and enabling their use in non-generic applications [1]. Enzymes also are potential as electric power sources in implantable devices in living organisms due to its attractive property of operating under physiological conditions [2]. The enzymes themselves, as well as their reaction products, can also be considered as relatively safe compared to non-biotic catalysts [1].

The relatively high concentration of glucose in blood (5-8 mM) leads the glucose/O_2 enzymatic fuel cell (EFC) such as glucose oxidase (*GOx*) has received

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most attention [3]. The applications of *GOx* onto electrode surfaces for sensors and biofuel cells need the development of a functional immobilization system. For these features, the efficient electron transfer achievement between the enzyme active centre and the electrode is crucial that usually requires a mediated electron transfer (MET) mechanism [4]. The function of these mediators to transfer an electron more rapidly (in solution) to the electrode surface [5]. The small redox active particles and polymers are often used as electron transfer carriers (mediators), such as organic dyes, ferrocene and its derivatives, modified vitamin complexes and conducting salts [4].

To achieve efficient electron transfer, the use of *GOx* has been often combined with mediator compounds as electrodes. Carbon paste modified by nanofiber-polyaniline is used in this study as mediator. Carbon is usually considered due to its biocompatibility, chemical stability and conductivity (not corrosive so that does not affect to the performance of the biological culture) [6]. Polyaniline is a common material for manufacturing electrodes such as biosensors, biofuel cells, and super capacitor applications in nano composite forms [7]. In this study, nanofiber-polyaniline has been synthesized in an emeraldine salt form by the interfacial polymerization method. The addition of polyaniline into the carbon paste aims to counteract the empty spaces amongst the graphite particles. Of course, it will improve the electricity conductivity on the electrode because the electron can be transferred continuously. In addition, the graphite can function to reduce friction so that the current endurance can be increase. This study reports for an efficient, simple and cost effective method for the functional immobilization of *GOx* onto nanofiber-polyaniline-modified carbon-paste-electrode surfaces which can act as bioanodes in fuel cell applications. In particular, *GOx* is immobilized onto the electrode surface using glutaraldehyde as a linker molecule.

EXPERIMENTAL SECTION

Materials

Glucose Oxidase (GO*x*) from *Aspergillus niger* (SIGMA), aniline, $(NH)_4)S_2O_8$, HCl, toluene, double distilled water, paraffin, graphite powder, glutaraldehyde, acetate buffer, glucose, KCl, $K[Fe(CN)_6]$, teflon tubes, Pt, Cu, and Ag/AgCl electrodes.

Instrumentation

Centrifuge (Beckman USA Mode J2-21), eDAQ potentiostat-galvanostat and SEM (Scanning Electron Microscopy).

Procedure

Nanofiber polyaniline synthesis

The nanofiber polyaniline was synthesized with interfacial polymerization between organic and water phases as previously described [8]. The organic phase (1.0 M aniline in toluene) was mixed without stirring with the water phase (HCl-$(NH)_4S_2O_8$) and then incubated overnight. The sediments (polymers) which formed were rinsed seven times and harvested by centrifugation using double distilled water. After that, the polymers were stored in a desiccators before analysis using Scanning Electron Microscopy (SEM).

Carbon paste electrodes fabrication

Carbon paste electrodes (CPEs) were fabricated as previously reported Colak et al. [9] by mixing 100 µL of paraffin and 0.15 g of graphite powder. In order to modify the carbon paste electrodes (MCPE), 2 mg of polyaniline was added to the mixtures of paraffin and graphite powder. Then, each mixture was deposited at a height of 0.7 cm on teflon tubes (0.8 cm diameter and 3.0 cm length). The electrode surfaces were made smooth using oil-paper.

Cyclic voltammetry measurement

Cyclic voltammetry was carried out with a eDAQ potentiostat-galvanostat, using both Carbon Paste Electrodes (CPE) and Modified Carbon Paste electrodes (MCPE), Ag/AgCl (3.0 M KCl) and platinum wire were used as working, reference electrodes and counter electrodes, respectively.

Immobilisation of GOx onto MCPE

Glucose oxidase (*GOx*) from *Aspergillus niger* (analytical grade) was purchased from Sigma-Aldrich. *GOx* was immobilized onto MCPE by using glutaraldehyde as a crosslinker [9]. Briefly, 50 µL of *GOx* (5204.3 U mL^{-1}), 1.0 mg of bovine serum albumin and 30 µL of 2.5% glutaraldehyde were added to a 50 µL aliquot of 100 mM acetate buffer, followed by gentle shaking. The solution was then dropped onto the surface of electrodes and then dried in an ice bath (approx. 4 °C). The resulting modified electrodes (*GOx*/MCPE) were removed and rinsed with 100 mM acetate buffer, and then stored at 4 °C in the 100 mM buffer acetate. The design of *GOx*/MCP bioanode is illustrated in Fig. 1.

Optimal pH and temperature profiles of GOx/MCPE

To establish optimal pH and temperature profiles, the *GOx*/MCPE synthesis was carried out over a range of different pHs (using 1 mL of 100 mM acetate buffer pH 4.0, 4.5, 5.0, 5.5 and 6.0) and temperatures (incubating at 20, 35, 45, 55, 65 and 75 °C). The cyclic

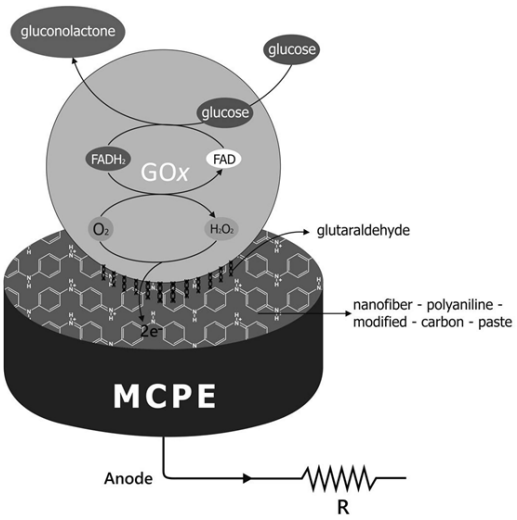

Fig 1. Schematic representation of the assembled *GOx* bioanode with nanofiber-polyaniline-modified-carbon-paste as an electron mediator

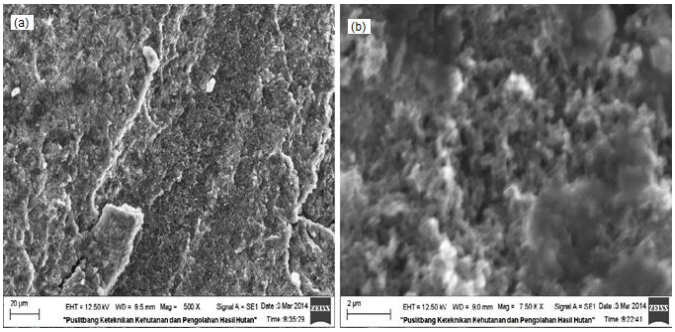

Fig 2. Polyaniline morphology by SEM with (a) 500 and (b) 7500 times magnification

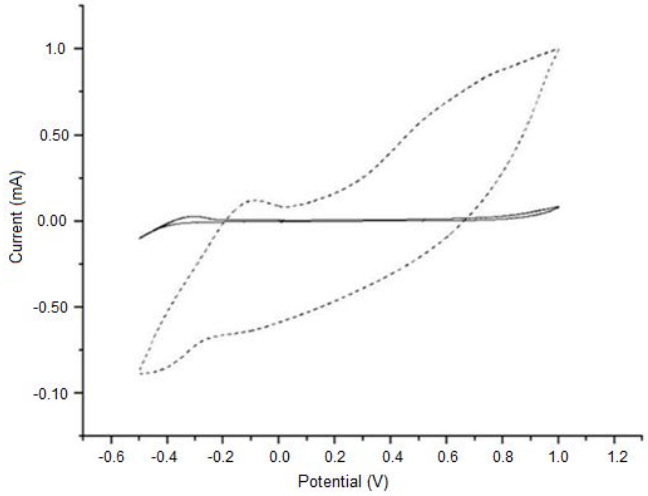

Fig 3. Cyclic voltamograms of CPE (black line) and MCPE (dashed line) at scan rate 100 mVs^{-1}, Ag/AgCl as reference electrode, cell conditions: 100 mM acetate buffer, 250 mM glucose, room temperature, 100 mM $K[Fe(CN_6]$

voltammetry was measured in the electrochemical cells by placing electrodes (*GOx*/MCPE), reference electrodes (Ag/AgCl) and counter electrodes (Pt) in a 100 mM $K[Fe(CN)_6]$ aqueous solution containing 15 mM glucose as the substrate.

Kinetic assay of GOx/MCPE

Initial reaction rates of *GOx* activity were determined at different substrate concentrations ranging from 12 to 480 µM glucose/ml of 100 mM acetate buffer, pH 4.5 at room temperature. The kinetic constants K_m and V_{max} were estimated following the method of Lineweaver and Burk [10] by measuring cyclic voltammetry in electrochemical cells as described above.

RESULT AND DISCUSSION

Synthesis and Morphology of Nanofiber Polyaniline

There was a separation of two phases between organic (aniline in toluene) and water (HCl and HCl-$(NH)_4S_2O_8$) when polyaniline was synthesized. While the organic phase was on the top layer (orange), the water phase was below (white). The polymerization could be seen by the formation of teal compound (emeraldine salt) between the two phases. The salt has hydrophilic groups which actively diffuse into the water phase [11-12]. The complete polymerization was identified by larger numbers of polymers being formed giving a darker color (becomes dark olive). An amount of 400 mg of polyaniline was produced for each reaction vessel. In addition, polyaniline morphology analyzed by SEM with 500 and 7500 magnification, appeared as fibers and hollow spaces because polymers were bound to each other. The polyaniline size of each polymer was about 110-120 nm (Fig. 2). This material was used to produce MCPE.

Cyclic Voltammetry of CPEs and MCPEs

The performance of carbon-paste and modified-carbon-paste electrodes was assessed using CV analysis. The results are shown in Fig. 3. This compares the CV curves produced between CPE and MCPE without using *GOx*. Fig. 3 shows the current of the electrode as a function of the operating voltage (*I-V*). The maximum current is the highest difference between top and bottom lines of oxidation picks of the CV curves. As shown in Fig. 3 there was a higher difference of a pick height at -85 mV between dashed lines (in carbon-paste-electrodes modified by nanofiber-polyaniline, MCPE), compared to the unmodified ones (CPE) which had the highest difference

Table 1. Current and voltage of peak oxidation on CPE and MCPE

Electrode type	Maximum voltage (mV)	Maximum current (mA)
CPE	-304	2.76×10^{-2}
MCPE	-85	12.09×10^{-2}

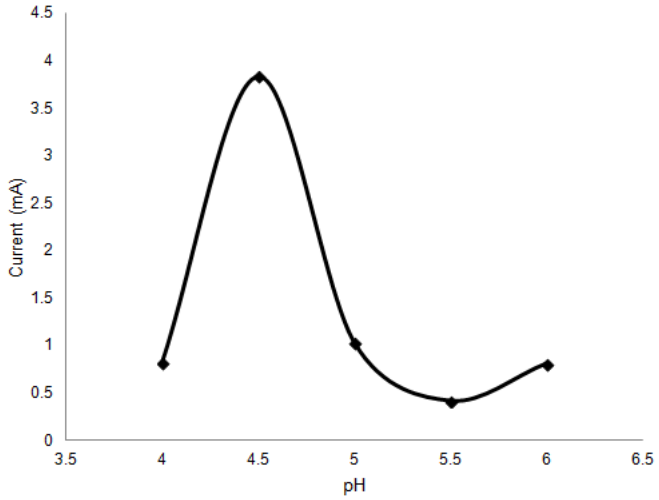

Fig 4. The current curve of the *GOx*/MCPE at pH 4.0-6.0, scan rate 100 mVs^{-1}. Cell conditions: 100 mM acetate buffer, 250 mM glucose, room temperature, 100 mM $K[Fe(CN)_6]$

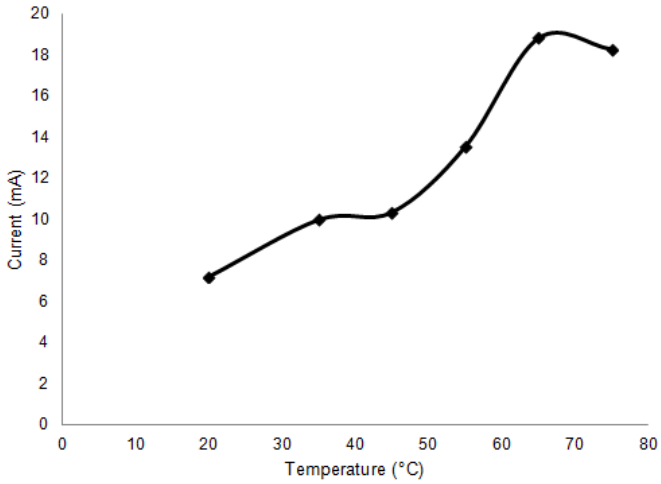

Fig 5. The current curve of the *GOx*/MCPE at temperature 20-80 °C, scan rate 100 mVs^{-1}. Cell conditions: 100 mM acetate buffer (pH 4.5), 200 mM glucose, 100 mM $K[Fe(CN)_6]$

of pick at -304 mV. The maximum currents of MCPE larger about 4.4 times than the CPE (are shown on Table 1). This result indicated that the modification of carbon paste with nanofiber-polyaniline (110–120 nm) was effective in increasing the electroconductivity of the electrodes. The electroconductive property of polyaniline is caused by protonation in strong acid media when oxidative polymerization occurs [13]. The nano-size and fiber form of the polyaniline particle also influence its electroconductiveness because they contributed to a larger surface area of the electrodes [14]. Therefore, nanofiber-polyaniline MCPE were used to study the *GOx* bioanode with immobilization using crosslinking with glutaraldehyde.

Optimal pH and Temperature Profiles of GOx/MCPEs

Slow-scan CV responses of *GOx*/MCP anodes in the presence of 15 mM glucose at pH 4.0, 4.5, 5.0, 5.5, and 6.0 are presented in Fig. 4. In addition, Fig. 5 shows the current of the bioanode as a function of the pH at room temperature. As shown in Fig. 5, the maximum steady-state current of 3.8 mA is observed at pH 4.5 for glucose oxidation at *GOx*/MCPE in acetate buffer. However, a previous study showed the optimal pH of *GOx*/MCPEs was 5.0 in 100 mM phosphate buffer [9]. The different buffer used might influence the optimal pH of the electrodes. In addition, the number of positive charges in polyaniline was also one of the factors which influenced the optimal pH, as shown by Keyhanpour et al. [15] who found that the optimal pH for glucose oxidation in polyaniline/*GOx* was at 7.0. In this study [15] the polyaniline was synthesized using 1.0 M H_2SO_4. Fabiano et al. [16] stated that the charge numbers of the polymer surface would affect the optimal pH of enzyme activity.

The *GOx*/MCP anodes were tested for temperature optimum using 20, 35, 45, 55, 65 and 75 °C in 100 mM acetate buffer (pH 4.5) and 12 µM of glucose to investigate optimal temperature for glucose oxidation at *GOx*/MCP anodes. Fig. 6 shows the current of bioanodes as a function of temperature. The results indicated that the optimal point was 65 °C. Then, after 65 °C the current decreased. The maximum current at 65 °C was 18.8 mA. Colak et al. [9] also showed the optimal temperature of *GOx*/MCPE was 65 °C in 100 mM phosphate buffer (pH 5.0).

The activation energy of the *GOx*/MCP anodes was also investigated by using the electrochemical version of Arhenius equation (Equation 1) [15]:

$$\ln I = \ln I_0 - Ea/RT \quad (1)$$

where I is the current observed at the certain temperature; I_0 is the initial current; R is the gas constant; T is temperature. The natural logarithm value of the current (ln I) was plotted versus the reciprocal of the temperature as shown in Fig. 6. The activation energy was calculated from the slope. This gave a value of activation energy (Ea) equal to 16.4 kJ mol^{-1}.

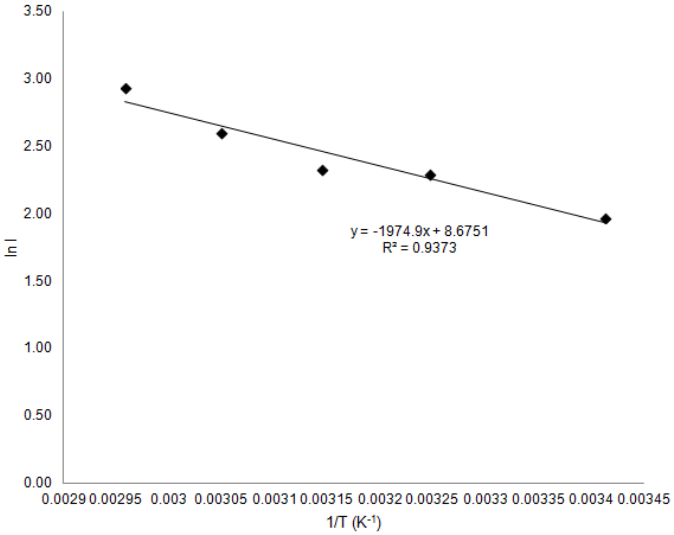

Fig. 6. The ln I plot versus 1/T

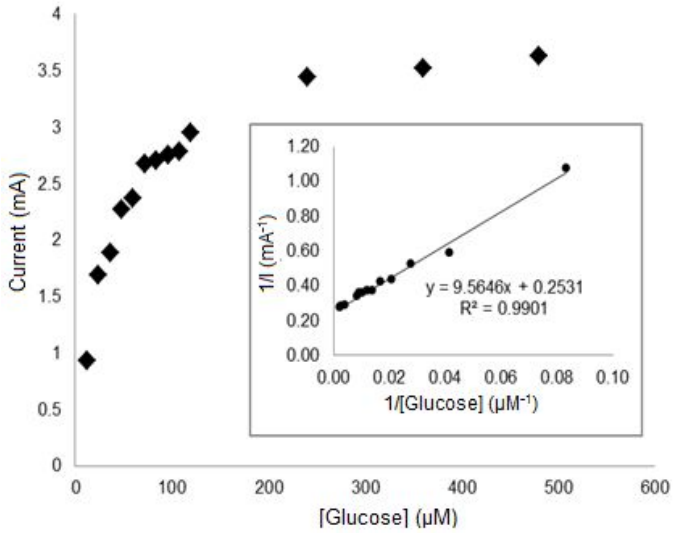

Fig 7. The current curve of the *GOx*/MCPE in the presence of various glucose concentrations in the electrolyte (range of 0.2 to 0.8 mM), scan rate 100 mVs^{-1}. Cell conditions: 0.1 M acetate buffer (pH 4.5), room temperature, 0.1 M $K[Fe(CN)_6]$

The value was smaller than the *GOx*/polyaniline electrodes in 100 mM acetate buffer pH 7 which had Ea equal to 49.5 kJ mol^{-1} [15]. This result clearly indicated that the *GOx*/MCP anodes had high catalytic performance as electrodes.

Kinetic Parameters of GOx/MCPE

The cyclic voltammetry response of the *GOx*/MCPE to increase concentrations of glucose at a constant potential of 1.0 V (*vs* Ag/AgCl) was investigated. A typical Michaelis-Menten kinetic trend was observed. This was characterized by a linear increase in the current output up to a concentration of 1.0 mM as shown in Fig. 7. The enzymatic affinity towards glucose was estimated in terms of the Michaelis-Menten constant (K_m) and was calculated by using electrochemical version of the Lineweaver-Burk equation of the enzyme kinetics (as shown in Fig. 7) [4]. The reciprocal of the current was plotted versus the reciprocal of the glucose concentration in the range of 12-480 μM. This gave values of K_m and I_{max} equal to 37.79 μM and 3.95 mA, respectively, whilst the K*m* was calculated from the slope (K_m/I_{max}) and the intercept ($1/I_{max}$) of the plot. The I_{max} of this study resulted higher value (3.95 mA) than *GOx*/polyaniline electrode in 100 mM acetate buffer pH 7 by Keyhanpour et al. (300 μA) [15].

The K_m value of *GOx*/MCPE was lower than *GOx*/MCPE from previous study (0.61 mM) [9] and much lower than one reported for the highly porous gold (hPG) electrodes with *GOx* (*A. niger*) immobilized by absorption (*GOx*/hPGE) (6.3±0.7 mM) [4], *GOx*/chitosan/ferrocene electrodes (1.5 mM) [17] and the native *GOx* from *A. niger* in solution (27 mM) [18]. This indicates that the immobilized *GOx* onto MCP electrodes has high enzymatic activity and higher affinity for glucose than *GOx* onto hPG electrodes and the soluble enzyme with a low diffusion barrier. In addition, the sensitivity value of glucose oxidation at *GOx*/MCPE anodes could also be estimated based on the function of current and glucoce concentration in a linear curve. The sensitivity value of our bioelectrodes was high enough (57.90 mA mM^{-1} cm^{-2}). The sensitivity of *GOx*/hPG electrodes was 22.7±0.1 μA mM^{-1} cm^{-2} [4].

CONCLUSION

Glucose oxidase immobilized onto nanofiber-polyaniline-modified-carbon-paste-electrodes by glutaral dehyde linker exhibits a high catalytic performances (energy activation 16.4 kJ mol^{1}), affinity for glucose (K_m value 37.79 μM) and maximum current (I_{max}) 3.95 mA. Sensitivity of these bioelectrodes (57.79 mA mM^{-1} cm^{-2}) was high enough compared to glucose oxidase attached to porous gold electrodes. In addition, the optimal pH and temperature of the *GOx*/MCPE were 4.5 (in 100 mM acetate buffer) and 65 °C, respectively. Future work will be aimed at the implementation of the *GOx*/MCP electrodes as an anode for enzymatic biofuel cell and in a microbiofuel cell for health care applications.

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